INFECTION AND IMMUNITY, May 1996, p. 1600–1608
0019-9567/96/$04.00+0

Vol. 64, No. 5

Defining Antibody Targets in *Streptococcus oralis* Infection

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Received 22 November 1995/Returned for modification 1 February 1996/Accepted 27 February 1996

Immunoblotting of sera from 12 neutropenic patients with *Streptococcus oralis* septicemia and 18 patients with endocarditis due to viridans group streptococci revealed immunodominant *S. oralis* antigens at 85 and 180 kDa. The former cross-reacted with a mouse monoclonal antibody to hsp90. The latter was identified by sequencing positive clones obtained by screening a genomic expression library of *S. oralis* with pooled sera from patients who had been infected with *S. oralis*. Antibody eluted from one of these clones reacted with the 180-kDa antigen of *S. oralis*. Southern blotting confirmed the origin of the clone from *S. oralis*. The derived amino acid sequence showed 76.2% homology with the PAc protein precursor of *Streptococcus mutans* and 73.8% homology with the SpaA protein precursor of *Streptococcus sobrinus*. Epitope mapping of the derived amino acid sequence with sera from patients with viridans group streptococcal endocarditis delineated nine epitopes. Peptides 1 (TMYPNRQPGSGWDSS) and 2 (WYSLNGKIRAVDVPK), representing two of these epitopes, and peptide 3 (YEVEKPLEPAPVAPS), representing the repeat proline region, were synthesized. These three peptides were used to screen a phage antibody display library derived from a patient who had recovered from *S. oralis* infection. Two of the human recombinant antibodies produced (SORAL 3 and SORAL 4 against peptide 3) and a human recombinant antibody (B3.7) against the conserved epitope (LKVIRK) of hsp90 gave statistically significant protection, compared with control groups, in a mouse model of lethal *S. oralis* infection.

Recent developments in antibody engineering have meant that it is now possible to extract the mRNA from human antibody-secreting cells, perform first-strand cDNA synthesis, and amplify the immunoglobulin genes encoding heavy- and light-chain variable domains by PCR (62). The heavy- and light-chain variable domains are then linked together to produce a human recombinant Fv fragment called a single-chain Fv fragment, or scFv (38). This is cloned into a phagemid vector so that the scFv is expressed fused to the viral coat protein, g3p, which occurs at the tip of the filamentous phage. Since the displayed antibody retains its antigen binding capability, it is possible to enrich for recombinant phage expressing high-affinity scFv. Such antibodies, if directed against microbial antigens associated with protective humoral immunity, are likely to have therapeutic value, particularly against those human pathogens for which antibiotic resistance is becoming a problem.

Viridans group streptococci are increasingly recognized as a cause of septicemia in cancer patients (6, 9, 11, 16, 28, 44, 51, 56, 60). They are associated with a syndrome which includes septicemia (sometimes a rapidly fatal form with shock) and the Adult Respiratory Distress Syndrome. Difficulties in the taxonomy of viridans group streptococci have led some authors to describe the causative agents as viridans group streptococci without determining individual species (11, 28, 44, 51, 60). Others have been more precise (2, 6, 9, 16, 56), and recently, the descriptions for *Streptococcus oralis*, *Streptococcus mitis*, and *Streptococcus sanguis* have been emended and *Streptococcus gordonii* has been described (29). *S. oralis* has been shown to be an important pathogen in neutropenic cancer patients (2) and in infective endocarditis (15). A recent survey of 47 strains of viridans group streptococci from 42 confirmed cases of infective endocarditis demonstrated that the most common species were *S. sanguis* (31.9%), *S. oralis* (29.8%), and *S. gordonii* (12.7%) (15). Penicillin tolerance has been reported among isolates of viridans group streptococci from cases of endocarditis (24, 59), and penicillin resistance has been reported among isolates associated with toxic shock syndrome (9, 11, 16, 51) and other infections (52, 53). Resistance in *S. oralis* is mediated by an altered penicillin binding protein 2B gene which is thought to have spread from *Streptococcus pneumoniae* (10). *S. oralis* is genetically related to *S. pneumoniae*, as the 16S rRNA sequences exhibit a more than 99% homology (25) and both species contain ribitol and choline in their cell walls (30).

S. oralis is therefore a significant human pathogen which may have decreased susceptibility to penicillin and could potentially be treatable with human recombinant antibodies. This would be dependent on obtaining high-affinity antibodies against defined microbial targets associated with protective immunity. The former is facilitated by obtaining the initial mRNA from a patient who has recovered from the infection and the latter is facilitated by defining discrete epitopes on immunodominant antigens. This approach was successfully used to produce protective scFv against *Candida albicans* heat shock protein 90 (hsp90) (40–43).

This paper describes the antibody response in 12 cases of septicemia due to *S. oralis*, in neutropenic patients with positive blood cultures who recovered following antibiotic therapy. Multiple sera taken during the course of the infection were immunoblotted against extracts of *S. oralis*, *S. gordonii*, and *S. sanguis* and compared with sera from patients with endocarditis due to viridans group streptococci and from patients with no laboratory evidence of streptococcal infection. This delineated two immunodominant antigens of *S. oralis* associated with an antibody response in patients recovering from the infection. One of these was conserved among all three species and reacted with a monoclonal antibody to hsp90 (40). A second antigen, with an apparent molecular mass of 180 kDa, was further characterized by cloning and sequencing. The antibody response was further defined to the epitope level by the Geysen technique (20), as previously applied to the PAc antigen of *Streptococcus mutans* (27, 39). The derived amino acid sequence was synthesized as a series of overlapping oligopep-

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tides on pins, and reactivity with patients' sera was assayed by a modified enzyme-linked immunosorbent assay (ELISA) (20). By comparing antibody-positive sera with antibody-negative sera, it was possible to identify immunodominant oligopeptides representing potential B-cell linear epitopes. Key epitopes, in the form of synthetic peptides, were then used to select scFv from a phage antibody display library derived from a patient with antibodies to *S. oralis* (38). These were then assessed together with scFv against hsp90 for their protective potential in a mouse model of the infection.

MATERIALS AND METHODS

Antigen preparations for immunoblotting. Antigen preparations were obtained from *S. sanguis* NCTC 7863, *S. oralis* NCTC 7864, and *S. gordonii* NCTC 7868 grown at 37°C in brain heart infusion broth and fragmented as previously described (7).

Sera examined by immunoblotting. (i) Group 1: septicemias. All patients (n = 12) were pyrexial and neutropenic, had severe oral mucositis and at least one set of blood cultures growing *S. oralis*, and subsequently responded to appropriate antibiotic therapy. At least two serum samples (pre- and postinfection) and up to four serum samples from each patient were examined. Four patients had chest X-ray changes indicative of the Adult Respiratory Distress Syndrome. Isolates were initially identified by Rapid Strep API 32 and confirmed by a reference center (J. Hardie, Royal London Hospital).

(ii) Group 2: endocarditis. All patients had clinical evidence of endocarditis, a positive echocardiogram, and positive blood cultures. Isolates were identified (Rapid Strep API 32) as *S. gordonii* (n = 14), *S. sanguis* (n = 2), and *S. oralis* (n = 2).

(iii) Group 3: controls. Forty serum samples from hospital inpatients with no clinical or laboratory evidence of ongoing streptococcal infection or endocarditis were examined; 20 of these came from neutropenic patients.

Patients' sera were examined at a dilution of 1:10 against immunoblots as described previously (7). Blots for which the antibody response was >50 mm by reflectance densitometry (Chromoscan 3; Joyce Loebel) were regarded as positive. When multiple sequential sera were tested, a constant result was recorded if the variation in height of the trace remained within 5 mm. A rising antibody titer was recorded if there was an increase of at least 30 mm in trace height. A new antibody titer was recorded if a band with a height of >50 mm appeared in later sera, having been absent in the earliest serum. Reactivity with a murine monoclonal antibody to a conserved epitope (LKVIRK) on hsp90 (40) was determined at a dilution of 1:100.

Preparation and screening of a genomic expression library of *S. oralis*. A genomic library was constructed in expression vector λgt11, essentially as described by Young and Davies (63). Chromosomal DNA, from a clinical isolate of *S. oralis*, was partially digested with *Eco*RI, and fragments in the size range of 2 to 9 kbp were cloned into the unique *Eco*RI site of λgt11. The library was screened with pooled sera (1:100) from patients with antibodies to *S. oralis* detected by alkaline phosphatase-conjugated goat anti-human immunoglobulin G (IgG) (1:5,000) (Sigma, Poole, United Kingdom). Lysogens were prepared from positive clones in *Escherichia coli* Y1089 as previously described (23). The reactivity of the resulting β-galactosidase fusion proteins was examined by immunoblotting with patients' sera containing antibodies to *S. oralis* (1:10) together with a monoclonal antibody to β-galactosidase (1:1,000) (Promega, Southampton, United Kingdom).

Characterization of positive clones and DNA sequencing. Antibodies in patients' sera (1:10) were affinity purified against positive recombinant plaques and the bound antibody; eluted with glycine buffer, pH 2.8; and screened against an immunoblot of *S. oralis* (63).

PCR with λgt11 forward and reverse primers was used to amplify insert DNA from the largest fusion protein. This was subcloned into the TA Cloning System (version 1.3; Invitrogen Corporation, Oxon, United Kingdom) prior to DNA sequencing by the chain termination method (Sequenase version 2.0 Kit; United States Biochemical, Cambridge, United Kingdom). The first set of annealing reactions was done with universal primers; subsequent primers were derived from the sequences obtained from both the coding and noncoding strands. The DNA sequence was analyzed by the SEQNET service (SERC; Daresbury Laboratory, Warrington, United Kingdom), using the OWL database (3). *S. oralis* DNA restricted with *Eco*RI, *Hae*III, *Rsa*I, *Ssp*I, and *Xma*I (Promega) was Southern blotted and probed with ^{32}P-labelled plasmid DNA derived from the sequenced clone to confirm its origin from *S. oralis*.

Epitope mapping. A series of overlapping nonapeptides covering the derived amino acid sequence were synthesized on polyethylene pins with reagents from an epitope scanning kit (Cambridge Research Biochemicals, Cambridge, United Kingdom) as described previously by Geysen et al. (20). Peptide 1 consisted of residues 1 to 9, peptide 2 consisted of residues 2 to 10, etc. The reactivity of each peptide with patients' sera (1:200) was determined for IgG by ELISA. Data were expressed as A_{405} after 30 min of incubation. Sera from patients with septicemia due to *S. oralis* (postinfection, n = 5), endocarditis due to viridans group streptococci (*S. sanguis*, n = 2; *S. oralis*, n = 2; *S. gordonii*, n = 4; and *S. mutans*, n = 2), and endocarditis due to *Enterococcus faecalis* (n = 2) and inpatient control sera (n = 5) were examined.

Preparation of phage antibody display library and scFv. The phage antibody display library and scFV were produced essentially as previously described (42, 43). Briefly, peripheral blood lymphocytes were obtained from a patient who had recovered from *S. oralis* infection, by separation of 20 ml of heparinized blood over Ficoll. mRNA was extracted by guanidinium thiocyanate; this was followed by purification on an oligo(dT)-cellulose column (Quick Prep mRNA; Pharmacia, St. Albans, United Kingdom). First-strand cDNA synthesis was performed with a constant region primer for all four subclasses of human IgG heavy chains (HuIgG1 to -4) (38, 42), using avian myeloblastosis virus reverse transcriptase (HT Biotechnology, Cambridge, United Kingdom). The heavy-chain variable-domain genes were amplified by primary PCRs with family-based forward (HuJH1 to -6) and backward (HuVH1a to -6a) primers. An *Sfi*I restriction site was introduced upstream to the VH3a back-generated product, prior to assembly with a diverse pool of light-chain variable-domain genes derived from the library of Marks et al. (38). The latter also introduced a linker fragment (Gly_4Ser_3) and a downstream *Not*I site. By use of the *Sfi*I and *Not*I restriction enzyme sites, the product was unidirectionally cloned into the phagemid vector pCANTAB 5 (Pharmacia). The ligated vector was introduced into *E. coli* TG1 by electroporation, and phages were rescued with the helper phage M13K07 (Pharmacia). To enrich for antigen-specific scFv, the phage library was panned against peptides representing two of the epitopes delineated by epitope mapping (peptide 1, TMYPNRQPGSGWDSS, and peptide 2, WYSLNGKIRAVDVPK) as well as a peptide representing the repeat proline sequence (peptide 3, YEVEKPLEPA PVAPS). Panning was performed in immunotubes coated with the corresponding peptide (42). Bound phages were eluted with log-phase *E. coli* TG1. After rescue with M13K07, the phages were repanned against peptide a further three times. *Bst*N1 (New England Biolabs, Hitchin, United Kingdom) DNA fingerprinting was used to confirm enrichment of specific scFv after successive rounds of panning.

Reactivity of the dominant scFv types was assayed by indirect ELISA. Plates were incubated overnight at 4°C with peptide (10 ng ml^{-1} in phosphate-buffered saline [PBS]), washed three times in 0.05% Tween–PBS and three times in PBS, blocked with 20% skim milk in PBS (M-PBS) for 2 h at 37°C, and then incubated with scFv-containing phage supernatants (in triplicate) for 3 h at 37°C. After repeat washings, the plates were incubated for 1.5 h at 37°C with anti-filamentous phage (pVIII) monoclonal antibody B62-FE2 (diluted 1:1,000 in 3% bovine serum albumin in buffered saline) kindly provided by B. Micheel, Max Delbruck Center for Molecular Medicine, Humboldt University, Berlin-Buch, Germany. Binding was visualized with horseradish peroxidase-conjugated anti-mouse IgG (1:1,000 in M-PBS) (Sigma) and ABTS (amino-di-3-ethylbenzohiazole-6-sulfonate; Sigma) and read at A_{405} after 30 min of incubation.

Assessment in an animal model. *S. oralis* was grown overnight in brain heart infusion broth at 37°C and washed in saline, and the concentration was determined by hemocytometer and by plating of dilutions on blood agar. Next, 5.7 × 10^9 CFU was injected as a 0.1-ml bolus into the lateral tail veins of BALB/c mice. Two hours after inoculation, randomized groups of animals were given intravenously, as a 0.2-ml dose, positive scFv-bearing phages SORAL 1 (against peptide 1), SORAL 2 (against peptide 2), and SORAL 3 and SORAL 4 (against peptide 3), B3.7 (against the conserved LKVIRK epitope of hsp90), negative control phage B3.1 (against a *Candida*-specific epitope on hsp90), and M13K07 helper phage. Phage doses were standardized at 5 × 10^8 PFU. The cause of death was confirmed by culturing livers and kidneys on blood agar at 37°C. The results were analyzed by the χ^2 test, and significance was defined by Fisher's exact test (two tailed; $P < 0.05$).

RESULTS

Immunoblotting. Immunoblotting revealed bands varying in apparent molecular mass from 25 to 192 kDa. In patients with *S. oralis* septicemia, bands of 51, 85, 140, and 180 kDa were the most commonly recognized, by >66% (Table 1). Recovery from infection on antibiotic therapy was associated with rising antibody to the 180-kDa antigen (Fig. 1). The same sera also detected immunodominant antigens of 47, 80, and 85 kDa in *S. gordonii* (Table 2) and of 40, 50, and 85 kDa in *S. sanguis* (Table 3). Hospital inpatient controls most commonly produced antibodies to the 51-kDa band of *S. oralis* (42%), the 47-kDa band of *S. gordonii* (42%), and a 75-kDa band of *S. sanguis* (57%), but most antibodies were produced by a minority of these patients.

Sera from patients with endocarditis recognized more bands than sera from those with *S. oralis* septicemia or inpatient controls. A combination of IgM and IgG against the *S. oralis* bands at 85 and 180 kDa occurred in both patients with *S. oralis* endocarditis. Both patients with *S. sanguis* endocarditis

TABLE 1. Immunoblot results of patients' sera against *S. oralis* NCTC 7864

Antigen apparent molecular mass (kDa)	No. of patients with indicated antibody											
	S. oralis septicemia (n = 12)				Endocarditis (n = 18)						Controls[a] (n = 40)	
	Constant antibodies		Rising or new antibodies		*S. oralis* (n = 2)		*S. gordonii* (n = 14)		*S. sanguis* (n = 2)			
	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
192	1	3	1	5	1	1	2	10	1	2	6	7
185		1	1	3		1		4	1	1	3	5
180		2	9	10	2	2	4	13	1	2	1	7
155		4				1		9		1		2
140	3	8	1	2	1	2	2	6			3	10
105		1				1	1	5		1	3	3
85		8	1	1	2	2	4	11	1	2	4	4
76	1	5	2	1		1	1	3		1	2	2
65				1	1			3				
51		11	2			1	6	3		1	1	17
46		1	1			2		3				1
35				1		1		1		1		

[a] Inpatients with no known infection.

TABLE 2. Immunoblot results of patients' sera against *S. gordonii* NCTC 7868

Antigen apparent molecular mass (kDa)	No. of patients with indicated antibody											
	S. oralis septicemia (n = 12)				Endocarditis (n = 18)						Controls[a] (n = 40)	
	Constant antibodies		Rising or new antibodies		*S. oralis* (n = 2)		*S. gordonii* (n = 14)		*S. sanguis* (n = 2)			
	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
185	1	7				2	2	5				
180		6				2	12	14	2	2	1	7
105	2	4				1		1			1	4
85	1	10			2	1	12	14	1	1	1	1
80	1	9					5	12	1	1	1	15
76							5	7	1	1	2	13
72				1	1	1	5	7		1	2	5
70		7				1	4	5	1	2		
65	1	4					9	13	1	2		2
58							1	9				
56							2	9		1		
52							1	8		2		
47		10		2		2	12	14	1	1	3	17
40		8		1			5	8	2	1		1
37							8	8	2	2		
35		2		1			3	10	1	1		
33							2	9				
30							1	11	2	2		
28							2	7		1		

[a] Inpatients with no known infection.

had IgM and IgG against *S. sanguis* bands at 40, 42, 47, 81, 83, 85, and 120 kDa. *S. gordonii* endocarditis was usually associated (12 or more of 14 patients) with both IgM and IgG to *S. gordonii* bands at 47, 85, and 180 kDa.

The monoclonal antibody against a conserved epitope on hsp90 reacted with the 85-kDa bands of *S. oralis*, *S. gordonii*, and *S. sanguis*.

Characterization of positive recombinant clones. Screening the *S. oralis* expression library with sera from patients infected with *S. oralis* gave 10 positive clones with fusion proteins ranging from 135 to 185 kDa. These reacted with both the screening serum and monoclonal antibody to β-galactosidase (Fig. 2). The phage encoding the largest fusion protein reacted with serum antibody against the 180-kDa antigen of *S. oralis*. Southern blots confirmed its origin from *S. oralis* (Fig. 3). DNA sequencing identified the entire 1.81-kb insert DNA which contained a single open reading frame (Fig. 4). Scanning the OWL protein sequence database with the derived amino acid sequence demonstrated that it had 76.2% homology with the *S. mutans* PAc protein precursor (26) and 73.8% homology with the SpaA protein precursor of *S. sobrinus* (5, 31). This homologous region included the repeat proline region common to this bacterial protein family (26). A 472-amino-acid subset showed an 86.1% homology with the agglutinin receptor precursor of *S. sanguis* (14).

Epitope mapping. Epitope mapping defined nine areas

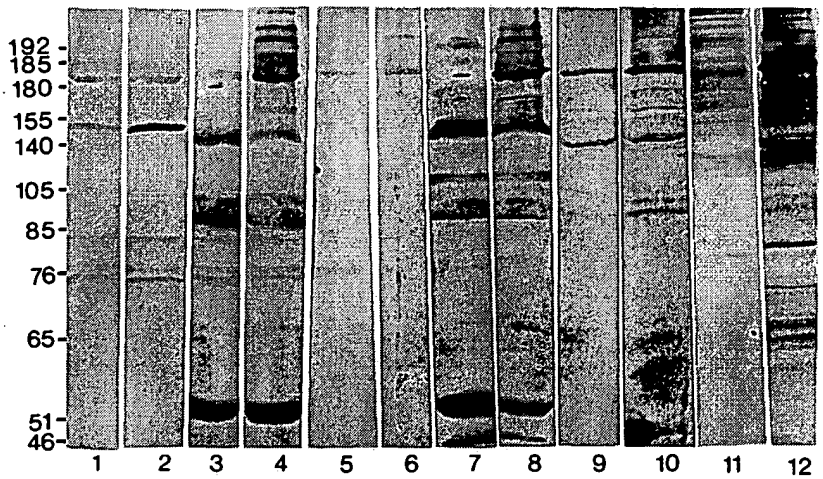

FIG. 1. Immunoblots of *S. oralis* NCTC 7864 with paired sera from three patients with *S. oralis* infection. Case 1, pre- and postinfection sera showing IgM (lanes 1 and 2, respectively) and IgG (lanes 3 and 4); case 2, pre- and postinfection sera showing IgM (lanes 5 and 6, respectively) and IgG (lanes 7 and 8); case 3, pre- and postinfection sera showing IgM (lanes 9 and 10, respectively) and IgG (lanes 11 and 12). Numbers on the left are molecular masses (in kilodaltons).

TABLE 3. Immunoblot results of patients' sera against *S. sanguis* NCTC 7863

Antigen apparent molecular mass (kDa)	No. of patients with indicated antibody											
	S. oralis septicemia (n = 12)				Endocarditis (n = 18)						Controls[a] (n = 40)	
	Constant antibodies		Rising or new antibodies		*S. oralis* (n = 2)		*S. gordonii* (n = 14)		*S. sanguis* (n = 2)			
	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
120		1				2	4	14	2	2	3	7
85		8				1		2	2	2	5	7
83									2	2		
81							2	8	2	2		
75	2	5			1	1	4	2		2	3	23
59					1	1		2	1		2	2
57					1	2	2	12	1		2	2
55					1	1				2	2	18
50		9										2
47							2		2	2	2	15
42					1		6		2	2		9
40		9							2	2		2
37		3									2	2
35									1			
25							6					2

[a] Inpatients with no known infection.

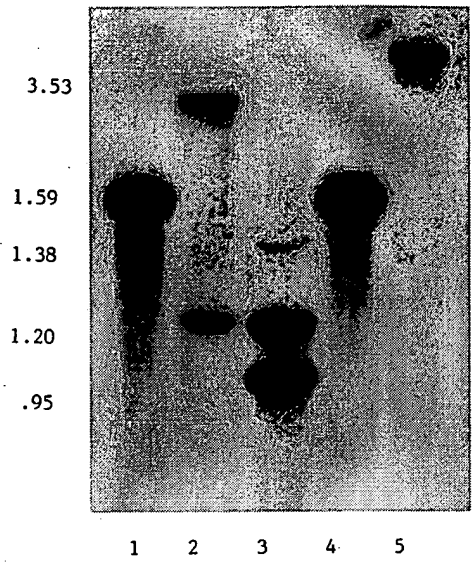

FIG. 3. Genomic *S. oralis* DNA. Genomic *S. oralis* DNA, after *Eco*RI, *Hae*III, *Rsa*I, *Ssp*I, and *Xma*I digestion (lanes 1 to 5, respectively), probed with the *S. oralis* cloned 1.81-kbp insert.

where patients with viridans group streptococcal endocarditis produced three or more consecutive wells with a mean optical density (OD) at least 2 standard deviations above that of inpatient controls (Table 4). Sera from all of the endocarditis patients recognized each epitope, with the exception of serum from one patient with *S. mutans* who was negative with AGRPK. This level of reactivity was not achieved by any of the sera from the patients with *S. oralis* septicemia or *E. faecalis* endocarditis. Epitopes SWYGAG, GKIRAV, and YPTVV were conserved with the PAc antigen of *S. mutans* and the SpaA antigen of *S. sobrinus*, while most of the other epitopes had similar, but not identical, sequences. RQPG was the exception, being unique to *S. oralis*. Peptides 1 and 2, representing unique (RQPG) and conserved (GKIRAV) epitopes, were synthesized. The proline repeat sequence identified within the cloned fragment (Fig. 4) was universally negative by epitope mapping but was synthesized (peptide 3) because pin technology may be inadequate at presenting proline-rich epitopes in an antigenic configuration (19).

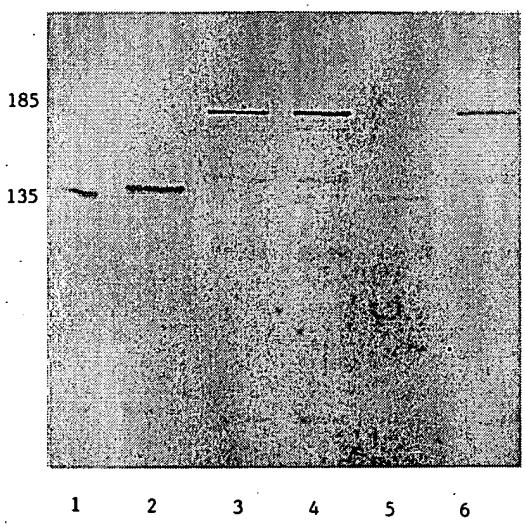

FIG. 2. Immunoblots of six of the recombinant fusion proteins showing reactivity with the monoclonal antibody specific to β-galactosidase. Numbers on the left are molecular masses (in kilodaltons).

Human recombinant antibodies. These peptides were used to pan the phage antibody display library. Primary PCR amplification of the families of heavy-chain variable-domain genes showed amplification of VH3a alone, producing a 330-bp product which was assembled with the light-chain variable-domain gene library. *Bst*NI fingerprints of the PCR-amplified scFv inserts before panning showed a highly heterogeneous library (Fig. 5). After panning against peptide 1, one *Bst*NI fingerprint predominated (about one in every two phages) and was the only type to produce a positive reading in the indirect ELISA (OD, 0.276 to 0.318; control OD, 0.17). This scFv (SORAL 1) was selected for animal work. Focusing was less pronounced after panning with peptide 2, but five phages did produce a positive ELISA (OD, 0.235 to 0.304; control OD, 0.138), and one of these was selected for animal work (SORAL 2). In contrast, after panning against peptide 3, all phages examined were indistinguishable by *Bst*NI fingerprinting (Fig. 5) and positive by indirect ELISA (OD, 0.25 to 0.3; control OD, 0 to 0.17). Two clones (SORAL 3 and SORAL 4) were selected for animal work.

Assessment in animals. A statistically significant increase in the number of survivors was observed at 4 h in mice given SORAL 3 (P = 0.0095) and at 48 h in mice given B3.7 (P = 0.03) or SORAL 4 (P = 0.02) (Table 5). Control groups given no antibody (M13K07 helper) or an irrelevant antibody (B3.1) showed no benefit; SORAL 2 and SORAL 1 showed either no activity or failed to reach statistical significance.

DISCUSSION

This study examined the antibody response in 12 patients with *S. oralis* septicemia and in 18 patients with viridans group

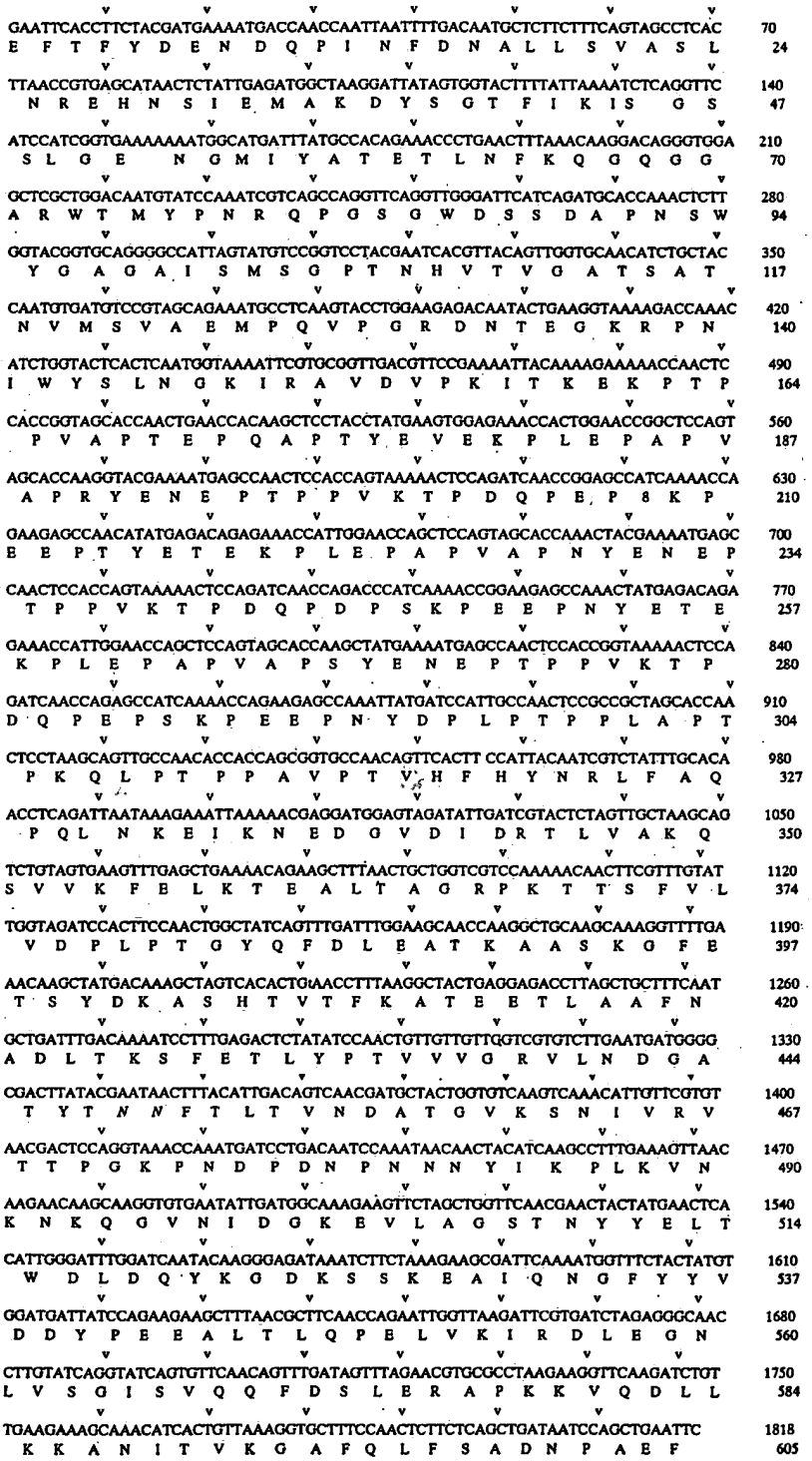

FIG. 4. DNA and amino acid sequences of the *S. oralis* homolog.

streptococcal endocarditis and started to characterize immunodominant antigens of *S. oralis* which may be associated with protective antibody. In patients with endocarditis, particular patterns of antibody response can be indicative of the causative bacterium (7), and the immunoblotting results reported here supported this, though the numbers for *S. oralis* and *S. sanguis* were too limited for generalizing. Two immunodominant antigens of *S. oralis* were characterized. The first was an 85-kDa

TABLE 4. Epitope map values for those wells where the mean OD was at least 2 standard deviations above that of the control. The overlapping amino acid sequences were derived by a comparison of first and last peptide sequences and were used to define the epitopes

Well no.	Epitope sequence	Value for[a]:				
		Hospital inpatient controls (n = 3)	Viridans group streptococcal endocarditis (n = 8)	*S. oralis* septicemia (n = 5)	*S. mutans* endocarditis (n = 2)	*E. faecalis* endocarditis (n = 2)
61	NFKQGQG	0.400 (0.027)	1.039 (0.353)	0.429 (0.200)	0.833 (0.125)	0.588 (0.054)
62	NFKQGQG	0.388 (0.034)	0.972 (0.347)	0.419 (0.230)	0.808 (0.122)	0.508 (0.027)
63	NFKQGQG	0.444 (0.058)	0.992 (0.329)	0.430 (0.215)	0.888 (0.195)	0.540 (0.013)
74	RQPG	0.341 (0.019)	0.902 (0.319)	0.322 (0.211)	0.839 (0.004)	0.407 (0.120)
75	RQPG	0.283 (0.033)	1.038 (0.470)	0.351 (0.180)	0.891 (0.011)	0.450 (0.119)
76	RQPG	0.282 (0.034)	1.053 (0.474)	0.455 (0.180)	0.917 (0.029)	0.493 (0.140)
77	RQPG	0.350 (0.045)	0.981 (0.358)	0.309 (0.070)	1.041 (0.043)	0.472 (0.130)
78	RQPG	0.280 (0.023)	0.918 (0.425)	0.307 (0.080)	1.142 (0.405)	0.423 (0.096)
79	RQPG	0.282 (0.015)	0.781 (0.312)	0.271 (0.091)	1.005 (0.418)	0.373 (0.084)
90	SWYGAG	0.308 (0.054)	0.852 (0.318)	0.287 (0.070)	1.027 (0.192)	0.378 (0.088)
91	SWYGAG	0.316 (0.069)	0.924 (0.340)	0.328 (0.080)	1.064 (0.138)	0.428 (0.136)
92	SWYGAG	0.344 (0.076)	0.917 (0.429)	0.298 (0.060)	0.914 (0.033)	0.377 (0.115)
93	SWYGAG	0.306 (0.007)	0.957 (0.378)	0.444 (0.140)	1.083 (0.225)	0.483 (0.131)
144	GKIRAV	0.306 (0.072)	0.824 (0.318)	0.348 (0.180)	0.779 (0.129)	0.389 (0.115)
145	GKIRAV	0.384 (0.071)	1.028 (0.309)	0.437 (0.210)	0.971 (0.199)	0.515 (0.120)
148	GKIRAV	0.303 (0.042)	0.852 (0.261)	0.348 (0.150)	0.881 (0.240)	0.438 (0.115)
147	GKIRAV	0.395 (0.035)	0.933 (0.285)	0.397 (0.180)	0.944 (0.258)	0.483 (0.131)
320	RLFAQPQ	0.392 (0.031)	1.147 (0.657)	0.491 (0.180)	1.010 (0.151)	0.494 (0.115)
321	RLFAQPQ	0.453 (0.049)	1.209 (0.417)	0.548 (0.230)	1.284 (0.300)	0.649 (0.171)
322	RLFAQPQ	0.467 (0.124)	1.145 (0.356)	0.531 (0.190)	1.289 (0.346)	0.593 (0.116)
359	AGRPK	0.395 (0.043)	0.921 (0.295)	0.401 (0.200)	0.749 (0.177)	0.480 (0.040)
360	AGRPK	0.492 (0.065)	1.049 (0.374)	0.428 (0.240)	0.775 (0.030)	0.491 (0.062)
361	AGRPK	0.407 (0.032)	0.907 (0.318)	0.382 (0.190)	0.657 (0.094)	0.462 (0.067)
362	AGRPK	0.430 (0.008)	1.054 (0.325)	0.423 (0.230)	0.753 (0.091)	0.525 (0.082)
363	AGRPK	0.421 (0.042)	0.946 (0.279)	0.391 (0.021)	0.665 (0.088)	0.477 (0.083)
376	PTGYQFD	0.277 (0.035)	0.791 (0.479)	0.428 (0.080)	0.762 (0.190)	0.339 (0.095)
377	PTGYQFD	0.415 (0.039)	1.045 (0.427)	0.359 (0.140)	1.548 (0.728)	0.487 (0.155)
378	PTGYQFD	0.351 (0.062)	0.802 (0.290)	0.291 (0.140)	1.274 (0.793)	0.380 (0.151)
426	YPTVV	0.309 (0.081)	0.852 (0.391)	0.269 (0.100)	1.275 (0.617)	0.410 (0.147)
427	YPTVV	0.215 (0.084)	0.473 (0.161)	0.225 (0.050)	0.525 (0.117)	0.289 (0.030)
428	YPTVV	0.325 (0.013)	1.170 (0.457)	0.450 (0.180)	1.143 (0.125)	0.561 (0.194)
429	YPTVV	0.339 (0.046)	0.964 (0.336)	0.413 (0.150)	1.050 (0.179)	0.488 (0.165)
430	YPTVV	0.349 (0.088)	0.976 (0.328)	0.465 (0.140)	1.002 (0.198)	0.458 (0.139)
577	LLKKA	0.279 (0.042)	0.863 (0.302)	0.353 (0.190)	0.681 (0.142)	0.466 (0.081)
578	LLKKA	0.349 (0.019)	0.907 (0.260)	0.410 (0.210)	0.808 (0.270)	0.557 (0.103)
579	LLKKA	0.351 (0.051)	0.937 (0.254)	0.415 (0.210)	0.882 (0.221)	0.549 (0.098)
580	LLKKA	0.395 (0.077)	1.035 (0.267)	0.423 (0.210)	0.898 (0.183)	0.587 (0.124)
581	LLKKA	0.331 (0.029)	0.847 (0.261)	0.581 (0.150)	0.843 (0.153)	0.482 (0.141)

[a] Data are OD values and standard deviations.

antigen present in *S. oralis*, *S. gordonii*, and *S. sanguis* which reacted with a monoclonal antibody to a conserved epitope (LKVIRK) on hsp90. The presence of antibody against fungal (8, 40, 41), bacterial (1), and malarial (4) hsp90 has been correlated with recovery from the corresponding infection. Both the mouse monoclonal antibody and a human scFv (B3.7) against the conserved LKVIRK epitope of hsp90 were protective in acute and chronic mouse models of disseminated candidiasis (40, 43). Here, preliminary animal data suggest that the same scFv (B3.7) might also be active against *S. oralis* (Table 5). If confirmed, they raise the possibility that such an antibody could be of therapeutic value to neutropenic patients in whom both fungi and streptococci are important pathogens.

A second *S. oralis* antigen of 180 kDa was identified by cloning and DNA sequencing. It was homologous to the PAc protein of *S. mutans* (26) and to SpaA of *S. sobrinus* (5, 31). Antibody against these two antigens has been previously identified in the sera of patients with *S. mutans* endocarditis (5, 54). PAc is a major cell surface protein involved in bacterial attachment which has been associated with protective antibodies against dental caries. Passive immunization with polyclonal antibodies against PAc, in bovine milk, prevented colonization by *S. mutans* in rats (45) and humans (17). Chicken egg yolk antibodies were protective against caries in rats (50). PAc-specific monoclonal antibodies, applied directly to the teeth, have been shown to prevent colonization by *S. mutans* in human and nonhuman primates (33, 36).

The immunoreactive *S. oralis* clone was homologous to

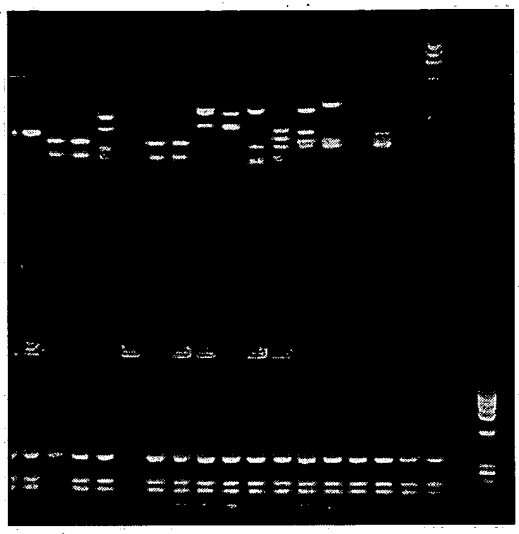

FIG. 5. *Bst*N1 fingerprints before (A) and after (B) panning against peptide 3.

amino acids 673 to 1274 from the PAc gene of *S. mutans* (26). Munro et al. (47) produced four overlapping fragments from the *S. mutans* gene by PCR and cloned and expressed them in *E. coli* cells. The recombinant, representing residues 816 to 1213, inhibited the adhesion of *S. mutans* to saliva-coated hydroxyapatite beads. It bound two of the monoclonal antibodies which had prevented oral colonization by *S. mutans* (33, 36) and was immunodominant in sensitized humans (27). *S. sobrinus* recombinants have been used to demonstrate that the 56-kDa region at the amino-terminal end of SpaA did not contain antigenic determinants or else that they did not fold correctly in the absence of the carboxy-terminal region (21). The immunodominant region overlapped the domain, conferring cross-reactivity with *S. mutans* (22). These observations are consistent with a key immunodominant area responsible for some of the functions of these proteins lying within amino acids 673 to 1274 from *S. oralis* and the corresponding regions in *S. mutans* and *S. sobrinus*.

In contrast, the alanine-rich amino-terminal region (the A region) from PAc in mice has been shown by epitope mapping to be immunodominant (49), under the control of the major histocompatibility complex class II genes (57), and capable of inducing a high serum IgG concentration when given intranasally coupled to the cholera toxin B subunit (58). An A region fusion protein was shown to competitively inhibit both the adherence of *S. mutans* to salivary agglutinin-coated hydroxyapatite and the fluid-phase agglutinin-mediated aggregation of this organism (12). A clone representing the amino-terminal 684 amino acids of SpaA was identified by screening a library with polyclonal rabbit antiserum against the PAc antigen of *S. mutans*. Synthesized peptides representing this sequence demonstrated that the alanine-rich area was immunodominant in mice (55). The binding of recombinant PAc fragments prepared in the expression vector pAX4a+ demonstrated that residues between 39 and 864 were important in binding to human salivary components (48). A peptide representing this area was able to inhibit salivary glycoprotein binding in a competitive adherence assay (46). When the PAc sequence was epitope scanned with sera from five adult subjects harboring *S. mutans* serotype C in their oral cavities (39), the immunodominance of the alanine-rich amino-terminal end was again confirmed. The homologous area within the *S. oralis* gene was absent from the clone sequenced here.

When the *S. oralis* sequence was mapped with endocarditis patients' sera, nine epitopes were delineated. Of these, SWYG AG, GKIRAV, and YPTVV (Table 4) were wholly conserved in the PAc antigen of *S. mutans* and the SpaA antigen of *S. sobrinus*. RQPG was unique to *S. oralis* and within a region which varied between different PAc sequences (26). This epitope was positive when the sera from the patients with *S. mutans* endocarditis were epitope mapped (Table 4). This is difficult to explain but may reflect a degree of conservation in the spatial complementarity between antibody and epitope, despite changes in the amino acid sequence. All the other epitopes had similar, but not identical, sequences in *S. mutans* and *S. sobrinus*.

Epitope mapping of the PAc antigen with sera from five patients colonized with *S. mutans* identified two epitopes within the region adjacent to the repeat proline area (39). One of these, KVTKEKP, had a similar sequence in *S. oralis*, KIT KEKP, while the other, VKPTAPTK, was unique to *S. mutans* (VAPTEPQA in *S. oralis*). Neither area was identified as an epitope in the present study. Kelly et al. (27), studying naturally sensitized humans, demonstrated immunodominant B-cell (residues 824 to 843) and T-cell (residues 803 to 822) epitopes which were separated in linear sequence from the adhesion epitopes (residues 1005 to 1044) of PAc. The present study failed to confirm the B-cell epitope but showed an antibody response to GKIRAV (residues 816 to 821) which is conserved between *S. oralis* and *S. mutans* and lies within the T-cell epitope. An immunodominant promiscuous T-cell epitope (residues 985 to 1004) was adjacent to an adhesion epitope (residues 1005 to 1024). The homologous area in *S. oralis* includes the B-cell epitope RLFAQPQ (residues 992 to 998) which was immunodominant in cases of endocarditis due to viridans group streptococci. Again, the B-cell epitope in endocarditis patients demonstrated here was present as part of an exclusively T-cell epitope in the naturally sensitized humans (27).

In vitro studies have indicated that during antigen processing, antibody may protect relatively large fragments of antigen from degradation (13, 37) and hence from association with major histocompatibility complex class II proteins. This would direct the T-cell response away from these B-cell-protected epitopes (61). This process may break down in endocarditis because the infection is chronic, resulting in the degradation of sufficient antigen to allow association with the major histocompatibility complex class II proteins. Adhesion epitopes overlapping with minor B- and T-cell epitopes (residues 1005 to 1054 and 1085 to 1134, respectively) in the PAc molecule were

TABLE 5. Results of in vivo assessment of the human recombinant antibodies. Negative controls were M13K07 phage and B3.1. Test antibodies were B3.7 and SORAL 1 to SORAL 4

Antibody	No. of mice	Survivors (%) at[a]:		
		4 h	24 h	48 h
M13K07 phage	15	47	20	7
B3.1	13	23	23	15
B3.7	13	46	46	46*
SORAL 1	15	40	40	40
SORAL 2	15	13	13	13
SORAL 3	9	100*	22	22
SORAL 4	17	47	47	47*

[a] *, statistically significant response ($P < 0.05$).

also reported (27). These incorporate the following epitopes from the current study: AGRPK (equivalent to *S. mutans* residues 1033 to 1037), PTGYQFD (equivalent to *S. mutans* residues 1048 to 1054), and YPTVV (equivalent to *S. mutans* residues 1099 to 1103) from *S. oralis*.

The repeat proline region did not produce detectable reactivity with sera by epitope mapping. This may reflect a lack of immunoreactivity or it could be because, as a secondary amine, proline imposes unique constraints on the conformation of the peptides containing it (19). As a result, for proline to be immunogenic it must be expressed within a framework larger than the short nonapeptides used for epitope mapping. This is likely to be especially true where multiple prolines are present. The proline-rich area within PAc has been implicated in spontaneous molecule self-aggregation (49). Gangloff et al. (18) demonstrated that a peptide representing this proline-rich region (peptide 3) was capable of inducing a significant antibody response in a rabbit which reacted with both the original peptide and the parent molecule. This area was shown to be independent from that which induced autoantibodies against human IgG (18, 46). However, in mice, a peptide derived from the proline-rich repeat area (residues 867 to 885) failed to elicit any antibody response and was not recognized by murine anti-recombinant PAc antibodies (58). Subsequently, peptide 3 was shown to contain at least one B- and one T-cell epitope (34). It induced peptide- and parent molecule-specific antibody responses without any carrier and stimulated the proliferation of rat lymph node cells primed with either free peptide or native SR protein. SR is the PAc homolog from *S. mutans* OMZ175 serotype f (46).

The animal studies (Table 5) suggested that SORAL 1, which is active against the *S. oralis*-specific epitope RQPG, may have had some activity at 48 h, but this was not statistically significant. SORAL 2, which is active against the conserved epitope GKIRAV, had no activity. Both SORAL 3 and SORAL 4, which are active against the repeat proline region, produced statistically significant results at 2 and 48 h (SORAL 3 and SORAL 4, respectively), suggesting that the repeat proline region may be a key target for protective antibody. These were human recombinant antibodies lacking the Fc region. The underlying mechanism could be that the antibody blocks microbial adherence. The $F(ab')_2$ fragment of a mouse monoclonal antibody to *S. mutans* PAc was as protective as the intact immunoglobulin in preventing recolonization with *S. mutans* in human volunteers (35). Alternatively, the scFv may induce active shedding of *S. oralis* PAc which would otherwise help the microbe avoid the immune defense system. PAc-specific polyclonal antibodies (IgG and secretory IgA) induced active secretion of *S. mutans* PAc, which could be a defense strategy for protecting the bacterium from antibodies (32).

In conclusion, the immunodominant 85-kDa antigen of *S. oralis* is probably a homolog of hsp90, while the 180-kDa antigen shows sequence homology to the *S. mutans* PAc protein and *S. sobrinus* SpaA. Human recombinant antibodies against the conserved LKVIRK epitope of hsp90 and the proline repeat region of the PAc protein homolog may have therapeutic potential, but further animal work is required to verify this. We are currently sequencing these scFvs, prior to subcloning them into a vector which will yield phage-free, soluble scFv suitable for further assessment in animal models of *S. oralis* infection.

ACKNOWLEDGEMENTS

W. Brooks was financially supported by a British Heart Foundation Fellowship.

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Editor: V. A. Fischetti